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14. ABSTRACT

Obesity, which induces low-grade inflammation, is a known risk factor for worse prognosis in many cancers including breast. We found that sphingosine-1-phosphate (S1P) produced by sphingosine kinases (SphKs) plays a critical role in obesity-related inflammation and breast cancer. Obesity increased S1P in the tumor microenvironment, as well as in the primary tumors. FTY720, a functional antagonist of S1PR1, dramatically decreased cancer progression by reducing expressions of SphK1 and S1PR1, and inflammatory cytokines including IL-6. Our results suggest a critical role for S1P in obesity-related inflammation and FTY720, an S1P axis inhibitor, appears to be a promising treatment for breast cancer in the obese condition, could be due to its effect on reactivate ERa expression and sensitize breast cancer cells to tamoxifen therapy.

15. SUBJECT TERMS

sphingosine kinase 1, sphingosine-1-phosphate, obesity, lung metastasis, macrophage, cytokines, ER□□□inflammation

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7. Participants & Other Collaborating Organizations…… N/A

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1. INTRODUCTION

The majority of breast tumors express the estrogen receptor α (ERα), which plays important roles in breast cancer pathogenesis and progression, and anti-estrogens, such as tamoxifen, are the first line of therapy(McDonnell and Norris 2002). Unfortunately, half of these patients will ultimately fail therapy due to de novo or acquired resistance as well as patients with ERα, progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2, also known as ErbB-2) triple negative breast cancer (TNBC), which is aggressive with high recurrence, metastatic, and mortality rates (Bayraktar and Gluck 2013). Epidemiological and clinical studies indicate that obesity, which is now endemic, increases breast cancer risk and is associated with worse prognosis , presenting with TNBC and endocrine therapy resistance (Pierobon and Frankenfeld 2013) .It has been proposed that chronic low grade inflammation evoked by obesity (Hotamisligil 2006) may play a role in aggravation of cancer progression by facilitating interactions between cancer cells and inflammatory stromal cells, such as macrophages (Khandekar, Cohen et al. 2011). Macrophage infiltration in the tumor microenvironment is recognized as an important enabler of cancer progression, and tumor associated macrophages (TAMs) correlate with increased angiogenesis, metastasis, and decreased survival of breast cancer patients (Sundaram, Johnson et al. 2013). There is growing evidence that sphingosine-1-phosphate (S1P), a pleiotropic bioactive sphingolipid metabolite formed inside cells by two closely related sphingosine kinases, SphK1 and SphK2, is involved in inflammation and cancer (Pyne and Pyne 2010, Spiegel and Milstien 2011). S1P regulates numerous cellular processes important for breast cancer, including cell growth, survival, invasion, lymphocyte trafficking, vascular integrity, angiogenesis, and cytokine and chemokine production, among others (Pyne and Pyne 2010, Spiegel and Milstien 2011). Although many of the actions of S1P are mediated by 'inside-out" signaling via its receptors, designated S1PR1-5 (Spiegel and Milstien 2011), our lab has demonstrated that SphK1 and intracellular S1P also play a direct role in TNF-α signaling and the canonical NF-kB activation pathway (Alvarez, Harikumar et al. 2010), important in inflammation and cancer. We recently showed that S1P produced by upregulation of SphK1 links chronic intestinal inflammation to colitis-associated cancer (CAC) and is essential for production of IL-6, persistent activation of NF-kB and STAT3, and consequent upregulation of one of its target genes, the S1P receptor, S1PR1 (Liang, Nagahashi et al. 2013). Our results suggest that SphK1 and S1P may also play similar roles in an animal model of breast cancer. Expression of SphK1 is elevated in patients with breast cancer (French, Schrecengost et al. 2003, Shida, Takabe et al. 2008) and correlates with poor prognosis (Ruckhaberle, Rody et al. 2008). Therefore, we believe that SphK1-S1P may have a critical role in obesity-related inflammation and that FTY720, an S1P axis inhibitor, could be a promising additional treatment for breast cancer in the obese condition.

2. KEYWORDS

sphingosine kinase 1, sphingosine-1-phosphate, obesity, lung metastasis, macrophage, cytokines, ERα, inflammation

3. ACCOMPLISHMENTS

The major goals of the project

Aim 1. Determine the role of SphK1 and S1P in obesity promoted low-grade chronic inflammation and tumor progression. (**Year 1**)

Aim 2. Dissect the cell-autonomous functions of the SphK1/S1P/S1PR1 axis in the primary tumor and in infiltrating myeloid cells in regulation of obesity promoted tumor progression and metastasis. (**Year 2, 3**)

Aim 3. Targeting the SphK1/S1P/S1PR1 axis to prevent elevation of SphK1 and S1PR1 and the NF-kB /IL-6/STAT3 amplification cascade, and reactivate ER expression in ER-negative breast cancer. (**Year 2, 3**)

ACCOMPLISHED

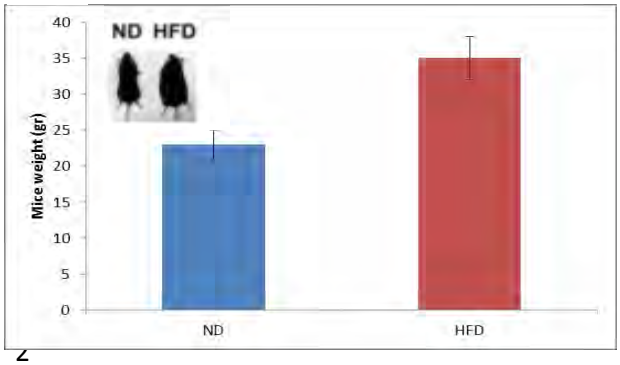
Major activities

1. Establishing the mouse models for the in vivo experiments described in Aim1.
2. Developing primary breast cancer model with relevance to human course of disease (primary site-induced lung metastasis).
3. Established in vivo techniques including tail vein injections of cancer cells in a lung metastasis model.
4. Establishing WT, SphK1$^{-/-}$ and SphK2$^{-/-}$ mice colonies.
5. Crossed SphK1$^{f/-}$SphK2$^{-/-}$ mice with transgenic mice expressing Cre recombinase under the control of the LysM promoter to generate LysM-Cre mice that specifically lack SphK1 in the myeloid lineage.
6. Establishing the use of E0771-luc breast cancer cells in a syngeneic breast cancer model to track primary tumor progression as well as metastasis in vivo.
7. Developed the use of FACS as a sensitive tool for dissecting the role the SphK/S1P axis in immune cells that participate in breast cancer and lung metastasis model.

Major findings

Our first goal was to determine the role of SphK1 and S1P in obesity promoted low-grade chronic inflammation and tumor progression. Because BALB/c mice are obesity resistant, in order to elucidate the effect of obesity on cancer progression and the link to SPHK1-S1P-S1PR1 axis, we used C57BL6 mice and E0771 breast cancer cells isolated from C57BL6 tumors. As expected, 8 weeks female mice fed with high fat diet (HFD) for 12 weeks developed severe obesity with an almost 2 fold increase of body weight compared with normal diet (ND) fed mice (Fig. 1A). E0771 mouse breast cancer cells were implanted into mammary fat pad of C57Bl/6 mice, which were fed with HFD or ND for 12 weeks prior to the implantation. Surprisingly, the mice fed with HFD did not develop larger tumors compare with those fed with ND (Fig. 1B). Recently there is growing evidence, as reported by NCI reports (2015) by many studies that have shown that overweight and obesity are associated with a modest increase in risk of **postmenopausal** breast cancer. This higher risk is seen mainly in women who have never used menopausal hormone therapy (MHT) and for tumors that express both estrogen and progesterone receptors. The increased risk of postmenopausal breast cancer is thought to be due to increased levels of estrogen from fat tissue in obese women (Roberts, Dive et al. 2010). **Overweight and obesity have**, **by contrast**, been found to be associated with a **reduced risk of premenopausal breast cancer in some studies** (Ballard-Barbash, Hunsberger et al. 2009). Our findings are in line with these studies. Our results suggest that in obesity related breast cancer study we should use mice models mimicking post-menopause that are with relevance to obesity-induced breast cancer.

A



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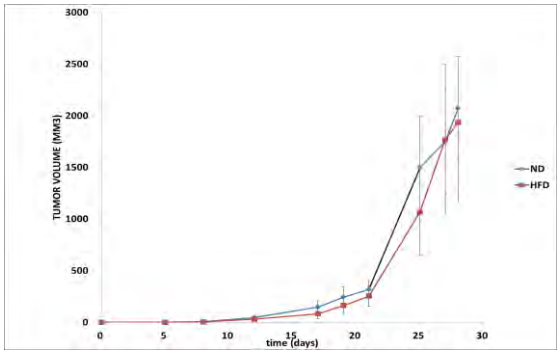

Figure 1. Effect of high fat diet on tumor growth in E0771 syngeneic breast cancer model. A, C57Bl/6 mice were fed with normal diet (ND) or high fat diet (HFD) and body weight was measured. B, C57Bl/6 mice were fed with ND or HFD for 12 weeks, and E0771 cells were implanted into the chest mammary fat pad. Tumor burden was measured during 28 after the implantation. N=15/group, 3 independent experiments

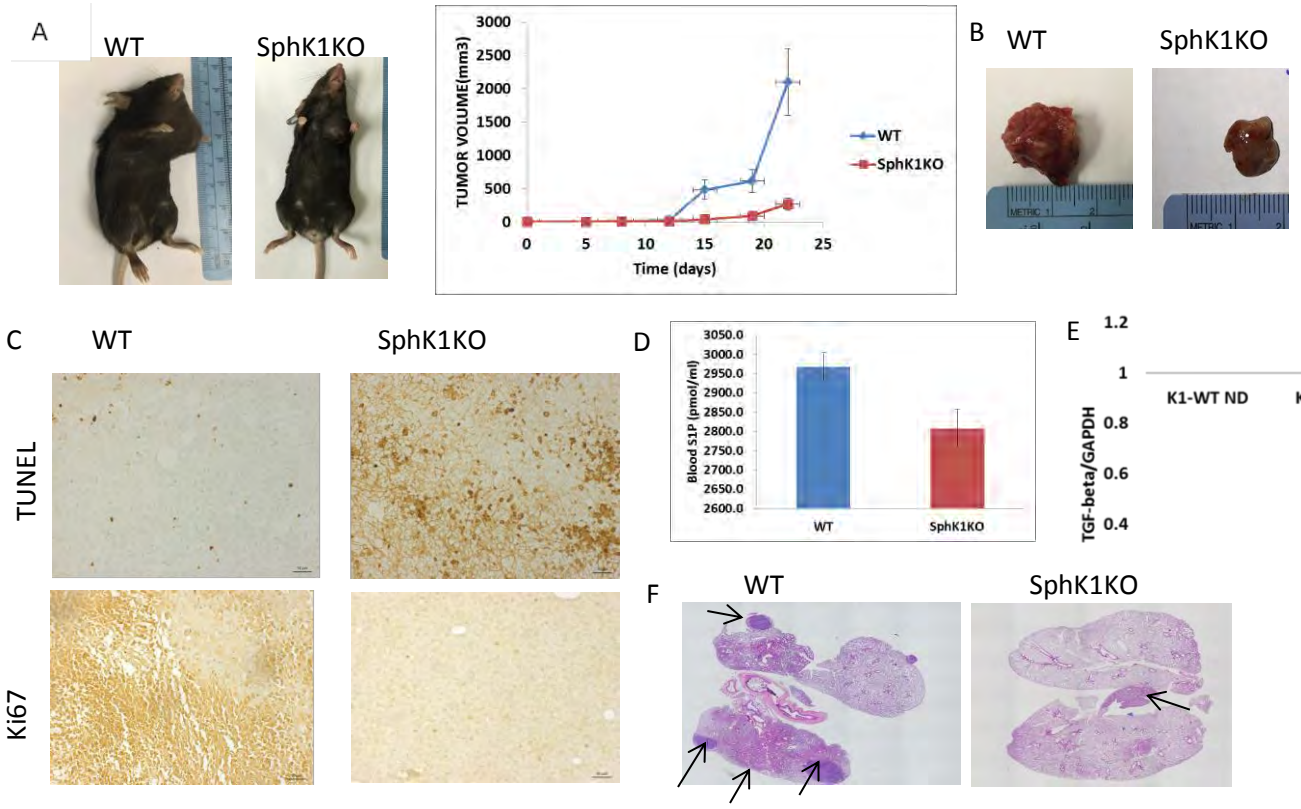

Figure 2. SphK1 has a role in breast-cancer progression and lung metastasis. E0771 cells were implanted into the 1st mammary fat pads. (A) Tumor volumes were measured daily. (B) Representative tumors. (C) Immunohistochemical staining of tumor sections for TUNEL and Ki67. (D) Blood S1P levels. (E) QPCR for TGF-beta mRNA expression in tumors.(F) Immunohistochemical staining of lung sections for HE. N=10

As reported in the previous report, we had setbacks regarding our WT and SphK mice colonies. During the past year we established these colonies in order to get preliminary results regarding the role of SphK1/S1P axis in breast cancer development and lung metastasis. To this end, we have been using the E0771 orthotopic model that closely mimics the progressive forms of estrogen-insensitive human metastatic breast cancer as a first approach. E0771 murine mammary cancer cells were orthotopically implanted into the 1st chest mammary fat pad of WT or SphK1KO (C57BL/6 background) female mice. In this enhanced metastatic breast cancer model, we found that in SphK1KO expressed less tumor burden (Fig. 2A) and lung metastasis (Fig. 2B) compare to the WT mice. Serum S1P levels in these mice were decreased (Fig. 2C). Epithelial-mesenchymal transition (EMT), a key step in the early stages of cancer metastasis, is orchestrated by several signaling pathways, including TGF-β/Smad signaling (Liu, Zeng et al. 2014). Interestingly, in tumors from SphK1KO expression of TGFb mRNA was significantly lower compare to Tumors from WT (Fig. 2D). In order to establish whether the reduced tumor burden and lung metastasis as shown in the SphK1KO mice is due to the effect SphK1-S1P axis might have on the immune cells in the micro environment, an ongoing animal using FACS analysis will be finished in the next 2 weeks.

S1P is generated by phosphorylation of Sph catalyzed by two isoforms of sphingosine kinases (SphK), type 1 and type 2. Since sphingolipid metabolism is often dysregulated in many diseases, targeting SphKs is potentially clinically relevant. While SphK1KO mice have lower circulating blood S1P compare to WT mice, SphK2KO have higher circulating S1P. To date, S1P is considered to have a cancer-promoting role (Pyne and Pyne 2010). Surprisingly, when we implanted E0771 cells into the mammary pad of WT and SphK2KO, SphK2KO mice showed reduced tumor burden and reduced lung metastasis compare to the WT mice (Fig 3A-C). SphK2KO had higher circulating S1P (Fig. 3D). These results suggest that S1P might have a protective role in this model and that SphK2 should be considered as a target for breast cancer therapy.

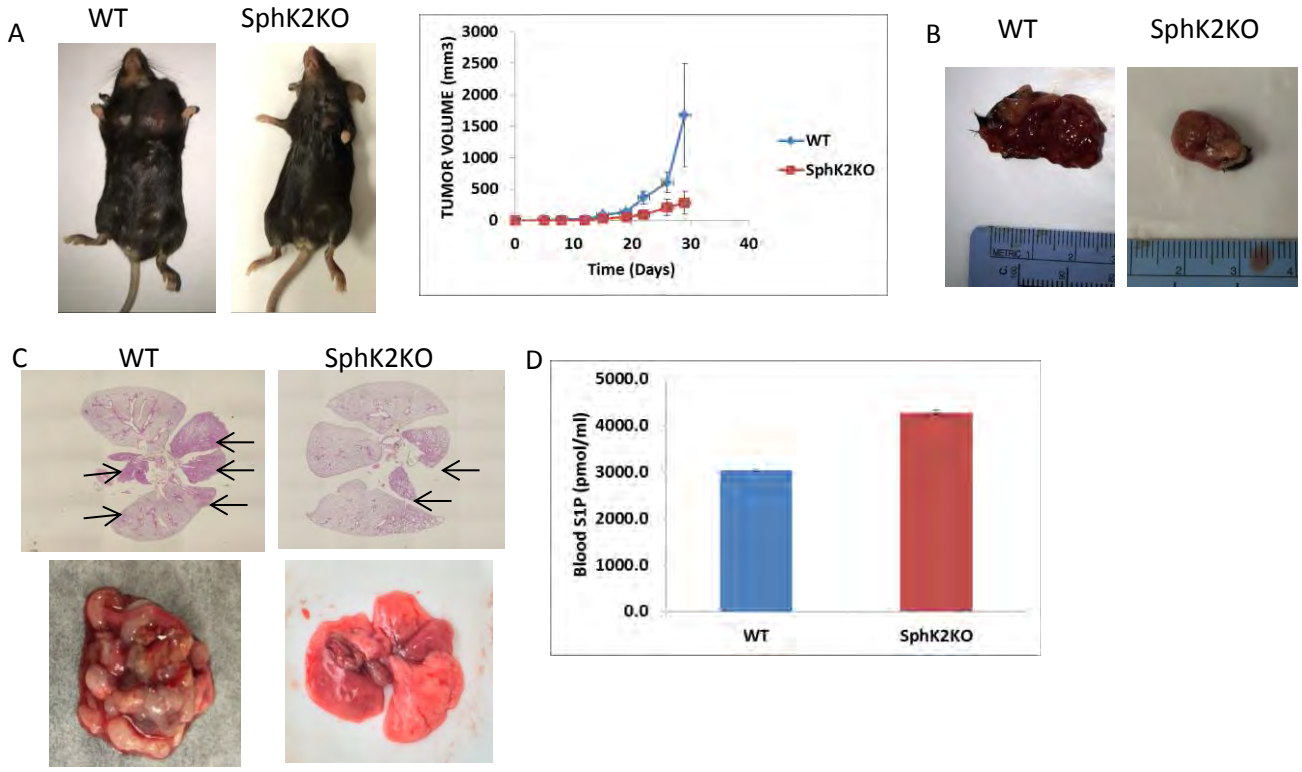

Figure 3 SphK2 has a role in breast-cancer progression and lung metastasis. E0071 cells were implanted into the 1st mammary fat pads. (A) Tumor volumes were measured daily. (B) Representative tumors. (C) Immunohistochemical staining of lung sections for HE. (D) Blood S1P levels. N=12

Conclusions:

Our initial results suggest a critical role for both SphK1 and SphK2 in breast cancer progression and lung metastasis. To date, S1P is considered to have a cancer-promoting role. Our results suggest that S1P might have a protective role in breast cancer. It could be due to different compartmentalization of S1P by differing localizations of SK1 and SK2 that enable their differential action on cancer cell. Recently it has been shown that S1P can affect the activity of immune cells such as macrophages polarization/population (Hughes, Srinivasan et al. 2008, Park, Lee et al. 2014). Taken together our results, we suggest that S1P affect different immune cells population that are recruited to the microenvironment and thus effect breast cancer

progression as well as lung metastasis. Our ongoing *in- vivo* experiment using FACS analysis will help to address this hypothesis.

Opportunities for training and professional development

1. VCU's Individual Development Plans (IDPs) provides me with a planning process that identifies both professional development needs and career objectives. This plan is helping me to pursue my long-term career goals and gives me the necessary tools to meet these goals. Also it is helping me to identify short-term goals and with a clearer sense of expectations and identification of milestones along the way to achieving specific objectives.
2. I am participating in the monthly VCU **Massey Cancer Center** (MCC) seminar series, one of only 67 cancer centers designated by the National Cancer Institute, both as an attendee and speaker, as well as the annual MCC Cancer Research Retreat that was held this year in June. This annual MCC retreat gives me the opportunity to meet in person key scientists such as Dr. Robert Schreiber who focused on molecular cell biology of cytokine receptor signaling and in defining the effects of signaling dysfunction on tumor development.
3. The numerous Massey Cancer Center core facilities I extensively use for my research. For example, the flow cytometry core has been invaluable in helping me develop cell sorting methodology for our studies.

How were the results disseminated to communities of interest
The results above are in preparation for a paper.

The plan for the next reporting period

1. Using the new breeding mice colony (SphK1 specific KO in the myeloid lineage) for breast cancer models as well as for a lung metastasis model to complete Aim1 and Aim2. Moreover, that will help us to deepen our understanding of the importance of the S1P-SphK1 axis in the immune cells that participate in cancer progression.
2. Establish post-menopause mice models and use them for obesity-induced cancer
3. Finish development of the E0771 SphK1 knockout cells using CRISPR/CAS and then use these cells in our breast cancer models.
4. I plan to continue the proposed research as originally suggested.

4. **IMPACT**
The impact on the development of the principal discipline of the project

We found that sphingosine-1-phosphate (S1P) produced by sphingosine kinases (SphKs) as well as both SphK1 and SphK2 play a critical role in breast cancer progression and lung metastasis. This may pave the way for development of new cancer therapeutics targeting this axis as a promising strategy for effective treatment of hormonal refractory breast cancer with available anti-estrogens. These treatments are critical for the prevention of the morbidity and mortality of breast cancer, and are even more important considering the increasing rates of obesity in the US.

The impact on other disciplines
Nothing to report
The impact on technology transfer
Nothing to report
The impact on society beyond science and technology
Nothing to report

5. CHANGES/PROBLEMS

During the past year I have created E0771-luc breast cancer cells in order to track them in a syngeneic breast cancer model to track primary tumor progression as well as metastasis *in vivo*. After cell implantation into the mammary pad as well as with IV cell injection for lung metastasis model, it appeared that the bioluminescence signal was low or not detected. Depilation-induced skin pigmentation in C57Bl/6 mice is a known occurrence, and presents a unique problem for quantitative optical imaging of small animals, especially for bioluminescence. As reported in several papers such as in Curtis A et al, the variability of skin pigmentation was found to drastically affect bioluminescent signal through the skin of the mice. When compared to signal through skin with no pigmentation, the signal through highly pigmented skin showed 90% signal reduction (Curtis, Calabro et al. 2011).
Our alternative approach will be to implant our 4T1-luc cells in BALB/C mice to induced breast cancer and treated with inhibitors such as Sphk1 inhibitor (SKI-1), S1PR1 inhibitor and to monitor the cancer progression as well as the lung metastasis.

6. PRODUCTS

The results above are in preparation for a paper.

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APPENDICES

CURRICULUN VITAE

NAME DORIT AVNI	POSITION TITLE POSTDOC

EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training).

INSTITUTION AND LOCATION	DEGREE (IF APPLICABLE)	YEAR(S)	FIELD OF STUDY
Tel Aviv University	BSc	1997	Biochemistry
Tel Aviv University	MS	2000	Cardiology
Tel Aviv University	PhD	2010	Immunology

Employment Experience

2012-present: Post-doctoral training, lab of Dr. Sarah Spiegel, Department of Biochemistry & Molecular Biology, Virginia Commonwealth University, Richmond Virginia

2011: Post-doctoral training, lab of Dr. Tsaffrir Zor, Biochemistry & Molecular Biology Department, Tel Aviv University

2008-2012: Scientific consultant, Allosterix-pharma Ltd., Yozmot Incubator, Emek-Hefer.

2005-2010: Ph.D. Research, lab of Dr. Tsaffrir Zor, Biochemistry and Molecular Biology Department, Tel Aviv University

2005-2011: Supervision of 8 undergraduate (project) students and 3 M.Sc. students

2005-2009: Teaching Assistant, Faculty of Life Sciences, Tel Aviv University

2003-2004: Research Assistant, ImmunoBar Ltd., Weizmann Institute (Rehuvot) + Sourasky Tel-Aviv medical center (Tel-Aviv). Establishment of a new biotech start-up as well as research focused on therapy for colon cancer.

2000-2002: Research Assistant, Prochon Biotech Ltd., Rehovot. My research focused on identifying and testing treatments for skeletal diseases and cancer

1997-2000: M.Sc. Research, lab of Prof. Uri Oron, Faculty of Life Sciences, Tel Aviv University

1997-1999: Teaching Assistant and supervision of undergraduate students, Tel Aviv University

1996-1997: Research Assistant, undergraduate project student, lab of Prof. Uri Oron, Faculty of Life Sciences, TAU. Clinical research in the field of cardiosurgery, using biochemical and statistical analysis.

Scholarships

2011: FEBS Scholarship for presentation in the 36th congress of the European Biochemical Societies, Torino, Italy

Conference Presentations

2015: Cancer research retreat, Massey Cancer Center, VA, USA
2015: Annual meeting of American Association for Cancer Research (AACR)
2014: Southeastern Regional Lipid Conference (SERLC)
2014: Cancer research retreat, Massey Cancer Center, VA, USA
2013: Cancer research retreat, Massey Cancer Center, VA, USA
2012: Cancer research retreat, Massey Cancer Center, VA, USA.
2011: The 36th congress of the European Biochemical Societies (FEBS), Torino, Italy
2011: The 6th congress of the federation of the Israel Societies for Experimental Biology (FISEB) Eilat, Israel.
2010: The Annual Meeting of the Israel Society for Biochemistry and Molecular Biology. Rehovot, Israel.
2009: Israeli Immunological Society 37th Annual Meeting.
2008: The 5th congress of the federation of the Israel Societies for Experimental Biology (FISEB) Eilat, Israel.
2004: Annual Meeting of the Israel Society for Biochemistry and Molecular Biology Tel-Aviv, Israel
2000: The congress of the Israel society of anti-oxidants, Bar-Ilan, Israel

Professional Associations

2012-present: Membership in American Association for Cancer Research
2004-2012: Membership in the Israel Biochemistry and Molecular Biology Association

Publications

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